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Selective Adhesion of Embryonal Carcinoma Cells and Differentiated Cells by Ca^{2+}-Dependent Sites

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The specificity of adhesion between embryonal carcinoma cells and fibroblastic cells of various origins was studied. Embryonal carcinoma cells have intercellular adhesion sites requiring Ca^{2+} (CDS). These sites were found to be sensitive to proteases but resistant to them in the presence of Ca^{2+}. CDS with a similar protease sensitivity is present in fibroblastic cells. When embryonal carcinoma cells of different lines were mixed, they adhered to each other nonselectively by CDS. Nonselective adhesion by CDS occurred also between fibroblastic cells of various lines. When embryonal carcinoma and fibroblastic cells were mixed, they preferentially adhered to homotypic cells. Fab fragments of antibodies raised against F9 cells (a nullipotent line of embryonal carcinoma) inhibited the adhesion between embryonal carcinoma cells but not between fibroblastic cells. This inhibitory activity of Fab was absorbed with embryonal carcinoma cells with CDS, but not with fibroblastic cells with CDS or embryonal carcinoma cells from which CDS was experimentally removed. SDS-polyacrylamide gel electrophoresis of radioiodinated cell surface proteins showed that the presence of a 140K-dalton component correlated with the presence of CDS in embryonal carcinoma cells, while the presence of a 150K-dalton component correlated with the presence of CDS in fibroblastic cells. These results suggest that CDS in embryonal carcinoma and fibroblastic cells comprise distinct molecules.

INTRODUCTION

Recognition between different cell types is probably a basic cellular function of morphogenesis in animal embryos. Pioneering works by Townes and Holtfreter (1955) demonstrated that the dissociated cells of amphibian embryos reaggregate and reorganize the tissue-specific structures through a process of segregating different cell types. Moscona (1956, 1957) found that cells dissociated from the tissues of avian and mammalian embryos become sorted out from each other when experimentally mixed. The molecular basis of such selective cell adhesions, however, has yet to be solved, although a number of hypotheses have been presented.

A series of works from our laboratory using cells of *in vitro* lines and embryonic tissues demonstrated that the adhesion between cells is mediated by at least two qualitatively different adhesion sites, Ca^{2+}-dependent site (CDS) and Ca^{2+}-independent site (CIDS) (Takeichi, 1977; Urushihara *et al.*, 1977, 1979; Ozaki *et al.*, 1978; Uéda *et al.*, 1980; Aoyama *et al.*, 1980; Takeichi, 1981) and this is supported by recent works carried out at different laboratories (Grunwald *et al.*, 1980; Magnani *et al.*, 1981; Thomas and Steinberg, 1981; Thomas *et al.*, 1981; Brackenbury *et al.*, 1981). CDS is very sensitive to trypsin but resistant to this protease in the presence of Ca^{2+}. CIDS requires a much higher concentration of trypsin to be inactivated, and Ca^{2+} has no effect in protecting this site against proteolysis. On the basis of such differences in the trypsin sensitivity between these two kinds of adhesion sites, it is possible for only one kind of site to remain intact in cells following treatment with trypsin under specified conditions. For example, Chinese hamster V79 cells which were dissociated with 0.01% trypsin containing Ca^{2+} retained only the CDS, while cells dissociated with 0.0001% trypsin without Ca^{2+} retained only the CIDS (Urushihara *et al.*, 1979). Molecules involved in the CIDS in V79 cells have already been identified (Urushihara and Takeichi, 1980).

The role of CDS and CIDS in cell recognition remains to be investigated. We showed that V79 cells adhere nonselectively to chick embryonic neural retina cells or to human retinoblastoma cells by CDS (Takeichi *et al.*, 1979; Uéda *et al.*, 1980), suggesting that this adhesion site does not have the function of recognizing different cell types. On the other hand, Grunwald *et al.* (1980) reported that chick embryonic neural retina cells with only CDS are capable of reorganizing tissue-specific structures in aggregates without protein synthesis. Their results suggest the possibility of the involvement of CDS in cell recognition which must be a key process in tissue reconstruction.

In our preliminary study (Atsumi and Uno, 1979), we examined the validity of the "dual adhesion sites hypothesis" in mouse embryonal carcinoma (teratocarcinoma) cells, and suggested that these cells have two kinds of adhesion sites which are similar in properties to CDS and CIDS present in other cell types. In the

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present work, we precisely analyze and compare the properties of CDS in embryonal carcinoma and differentiated cells, and explore the role of CDS in sorting out of embryonal carcinoma cells from differentiated cells. Since embryonal carcinoma cells resemble undifferentiated cells of early mouse embryos in several respects, it is expected that the conclusions obtained from the experiments using embryonal carcinoma cells can be extrapolated for understanding the adhesive properties of early mouse embryo cells.

MATERIALS AND METHODS

Cells. The multipotent embryonal carcinoma cell line AT805, which grows *in vitro* without feeder cells, was established from the embryoid body line SSEB (a subline of SEBIII isolated by Amano *et al.* (1978)) originally derived from OTT6050 (Stevens, 1970). Nullipotent embryonal carcinoma F9 (Bernstine *et al.*, 1973), mouse fibroblast W3 (Yanagisawa *et al.*, 1980), and mouse fibroblast L (a bromodeoxyuridine-resistant subline isolated by Murayama and Okada (1970)) were kindly provided by Dr. T. Matsuzawa, Dr. K. Yanagisawa, and Dr. Y. Okada, respectively. Mouse fibroblast 129F was established in our laboratory from a mass culture of dermal cells from the embryos of 129/Sv strain mice.

AT805 was maintained in an alpha medium (Stanners *et al.*, 1971) containing 8% fetal calf serum (FCS). Other lines and the Chinese hamster fibroblast V79 (Yu and Sinclair, 1964) were maintained in Dulbecco-modified Eagle's minimal essential medium (DMEM) with 6% FCS. Cells were passed by means of 0.1% crude trypsin (Difco, 1:250) and 1 m*M* EDTA in Ca^{2+}- and Mg^{2+}-free saline buffered with Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at pH 7.4 (HCMF, see Takeichi (1977) for ionic composition).

Cell dissociation. The monolayers of cells were rinsed three times in HCMF (with or without additional 1 m*M* $CaCl_2$, the former being used when the protease solution for cell dissociation contained Ca^{2+}). Protease dissolved in HCMF (with or without additional ions) (5 ml/10-cm plate) was then added. The plates were incubated for 20 min at 37°C on a gyratory shaker, and then the cells were collected and centrifuged. Following the use of trypsin, the cells were washed once with HCMF containing soybean trypsin inhibitor (1 mg/ml) (Sigma Chemical Co.) and twice with HCMF. After using other proteases, cells were washed three times in HCMF containing 1 m*M* $CaCl_2$ (HMF), incubated in 1 m*M* EDTA for 10 min in an ice bath, and then washed three times with HCMF. The cells were finally suspended in HCMF and dissociated by pipetting. All the steps above for washing the cells were carried out at 4°C. The viability of cells thus prepared was consistently higher than 90%, as estimated by the trypan blue exclusion test.

Some of the dissociation solutions are abbreviated as follows: 0.01% trypsin with $CaCl_2$, tryC; 0.01% trypsin with EGTA (ethylene glycol bis(aminoethyl ether) tetraacetic acid), tryE; 0.01% Pronase with $CaCl_2$, proC; 0.01% Pronase with EGTA, proE. Crystallized trypsin (10,700 BAEE units/mg; Sigma, type I) was used throughout the cell aggregation experiments. Pronase (1,000,000 tyrosine PU/g) was obtained from Kaken Chemical Company, Tokyo.

Cell aggregation. Aggregation of dissociated cells was carried out in the same manner as described previously (Urushihara *et al.*, 1979). Briefly, cells (1×10^5) in 0.5 ml HCMF with and without additional substances to be tested were placed in each well of a Linbro multiwell plate (No. 76-033-05) precoated with bovine serum albumin, and incubated at 37°C on a gyratory shaker. The degree of cell aggregation is represented by an index N_t/N_0, where N_0 is the total particle number prior to the initiation of aggregation and N_t is the total number of particles at incubation time t (min) (Takeichi, 1977). The data on cell aggregation shown in the results represent the mean of the values obtained from triplicate samples, unless otherwise noted.

Heterotypic cell aggregation. Cells stained with fluoresceine isothiocyanate (FITC) were mixed with unstained cells and allowed to aggregate, as described previously (Takeichi *et al.*, 1979). For microscopical observation, the cells were fixed by adding 1 vol of 10% paraformaldehyde to 1 vol of cell suspension in an ice bath. The fixed-cell suspension was placed on a slide glass and slightly squeezed with a coverglass. The samples were examined by an ultraviolet phase-contrast microscope (Olympus, Model BH-RFL).

Preparation of antibodies against F9. F9 cells were dissociated with 0.01% crystallized trypsin in the presence of 1 m*M* Ca^{2+} and suspended in HMF. Cells (1×10^8) mixed with Freund's complete adjuvant and the same number of intact cells without the adjuvant were injected at multiple subcutaneous sites in a rabbit. Thereafter, 2×10^8 intact cells dissociated as above without adjuvant were subcutaneously injected four times every 10 days. One week after the fifth injection, the antiserum (anti-tCF9) was collected. The Fab fragments of this antibody were prepared as described in our previous paper (Urushihara *et al.*, 1979) and the final preparations were dialyzed against HMF.

Radioiodination and electrophoresis. The surface proteins of dissociated cells were radiolabeled by a lactoperoxidase-catalyzed iodination as previously described (Takeichi, 1977). The labeled cells were dissolved in the sample solution with 2% sodium dodecyl sulfate (SDS)

for SDS-polyacrylamide gel electrophoresis (Takeichi, 1977) and boiled for 2 min following the addition of 2-mercaptoethanol (2% in final). The electrophoresis of the samples and the autoradiography of the gels were carried out as described previously (Urushihara and Takeichi, 1980).

RESULTS

Adhesive Properties of Embryonal Carcinoma Cells

Monolayers of AT805 cells were dissociated by various treatments and their aggregating properties studied. It was hard to dissociate the monolayers of AT805 cells completely into single cell suspensions with 0.01 to 0.1% trypsin containing 0.1 to 10 m*M* Ca^{2+} (tryC). The final cell suspensions always contained small cell clumps (about 50 cells at maximum). The reaggregation of such incompletely dissociated AT805 cells occurred only in the presence of Ca^{2+} (Fig. 1). The minimum concentration of Ca^{2+} required for the aggregation was on the order of 10^{-5} *M* (Fig. 2). This concentration was about ten times lower than that for the aggregation of V79 cells dissociated by identical treatment (Fig. 2). Monolayers of AT805 cells were dissociated completely into single cells by treatment with 0.01% trypsin in the presence of 1 m*M* EDTA or EGTA (tryE). These cells aggregated little either in the presence or absence of Ca^{2+} (Fig. 1).

The treatment of AT805 cell monolayers with 0.01% Pronase in the presence of 1 m*M* Ca^{2+} (proC) or the absence of this ion and the subsequent pipetting of the cell suspensions resulted in complete dissociation. Figure 1 shows that the cells dissociated with proC aggregate only in the presence of Ca^{2+}. The minimum concentration of Ca^{2+} required for the aggregation was about the same as that for the tryC-dissociated AT805 cells. The cells dissociated with Pronase in the absence of Ca^{2+} or in the presence of 1 m*M* EDTA or EGTA (proE) did not aggregate either in the presence or the absence of Ca^{2+}.

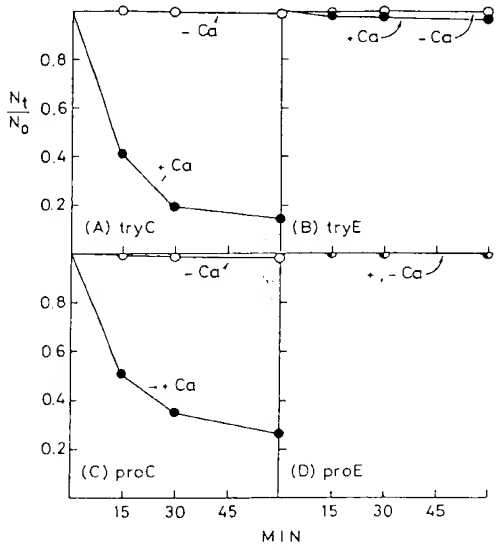

FIG. 1. Aggregation of AT805 cells dissociated by various treatments. Dissociated cells were incubated with and without 1 m*M* Ca^{2+} to bring about aggregation. (A) 0.01% trypsin with 1 m*M* Ca^{2+} (tryC), (B) 0.01% trypsin with 1 m*M* EGTA (tryE), (C) 0.01% Pronase with 1 m*M* Ca^{2+} (proC), and (D) 0.01% Pronase with 1 m*M* EGTA (proE).

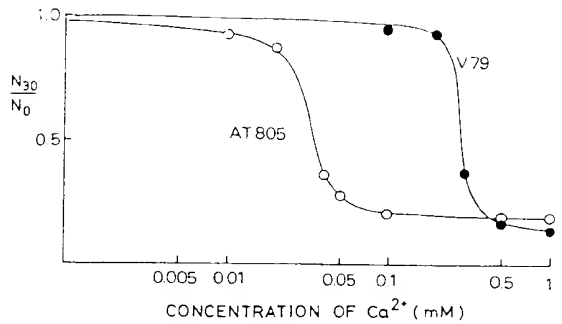

FIG. 2. Effect of different concentrations of Ca^{2+} on the aggregation of AT805 and V79 cells dissociated with 0.01% trypsin containing 1 m*M* (for AT805) or 0.2 m*M* (for V79) Ca^{2+}. Aggregation was measured after 30 min of incubation.

The minimum concentration of Ca^{2+} necessary to be added to trypsin or Pronase to obtain such cells that aggregate in the Ca^{2+}-dependent manner depended on the concentration of enzymes; the higher concentration of Ca^{2+} was required for the higher concentration of enzymes. For example, 0.1 m*M* Ca^{2+} was required for 0.01% Pronase, and 1 m*M* Ca^{2+} was required for 0.1% pronase. Mg^{2+} could not be substituted for Ca^{2+} in all the present systems.

These results suggest that AT805 cells have adhesion sites which are inactivated by proteases in the absence of Ca^{2+} but not in the presence of Ca^{2+}. Such sites are analogous to the Ca^{2+}-dependent sites (CDS) defined in other cell types (Takeichi, 1977). For further confirmation, tryC- or proC-dissociated AT805 cells were retreated with tryC, proC, tryE, or proE. As long as the second treatment with proteases was carried out in the presence of Ca^{2+}, the retreated cells always maintained the capacity for Ca^{2+}-dependent aggregation, whereas the cells retreated with proteases in the absence of Ca^{2+} lost this capacity for aggregation. Cells dissociated with tryE or proE recovered the potential for Ca^{2+}-dependent aggregation after 16 hr of monolayer culture. This indicates that the inability for aggregation of the tryE- and proE-treated cells is not due to cell death.

The same series of experiments as above was conducted on F9 cells. The results show that F9 cells have

TABLE 1
AGGREGATION OF CELLS OF VARIOUS LINES DISSOCIATED BY DIFFERENT TREATMENTS

	Aggregation (N_{60}/N_0)[b]									
	F9		V79		W3		129F		L	
Dissociation[a]	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
Trypsin + Ca^{2+}	0.15	0.98	0.19	1.02	0.33	0.98	0.20	1.00	0.72	0.95
Trypsin + EGTA	0.99	1.01	0.99	1.00	1.02	0.99	0.98	0.99	0.98	0.97
Pronase + Ca^{2+}	0.28	1.08	0.10	1.02						
Pronase + EGTA	0.95	0.99	1.01	1.00						

[a] Protease concentration was 0.01%. Concentration of Ca^{2+} added to trypsin was 0.2 m*M* for treating V79, 1 m*M* for F9, and 10 m*M* for W3, 129F, and L; that of Ca^{2+} to pronase was 1 m*M* for both of F9 and V79. EGTA was 1 m*M* in all the cases.

[b] Aggregation was measured following incubation with and without 1 m*M* Ca^{2+} for 60 min. Data represent the average of triplicate determinations.

essentially the same aggregating properties as AT805 cells (Table 1).

Ca^{2+}-Dependent Adhesion Sites in Various Fibroblasts

Aggregation of murine fibroblastic cells, W3, 129F, and L, dissociated with 0.01% trypsin in the presence and absence of Ca^{2+}, are shown in Table 1. As found in embryonal carcinoma cells, W3 and 129F cells dissociated with tryC showed Ca^{2+}-dependent aggregation, and cells dissociated with tryE did not aggregate. The minimum concentration of Ca^{2+} to be added to 0.01% trypsin in order to obtain the dissociated W3 and 129F cells having the capacity for Ca^{2+}-dependent aggregation was about 1 m*M*. The higher concentrations of Ca^{2+} (up to 10 m*M* examined) added to trypsin yielded cells having higher aggregating capacity. Under these dissociation conditions, cells became single. L cells dissociated with 0.01% trypsin in the presence of 1 to 10 m*M* Ca^{2+}, aggregated weakly only in the presence of Ca^{2+} (Table 1). The dependence of the aggregation of these fibroblastic cells on Ca^{2+} concentration was similar to that of V79 cells.

The effects of several proteases other than trypsin on CDS of fibroblasts were observed with V79 cells. Cells dissociated with Pronase (Table 1), papain (Worthington), and proteinase K (Merk) both in the presence and absence of Ca^{2+} showed essentially the same aggregating properties as those dissociated with trypsin under equivalent conditions.

Sorting out of Embryonal Carcinoma Cells from Fibroblasts

AT805 cells differentiate into a variety of tissues after developing into tumors in histocompatible mice. When the tumors were dissociated and cultured *in vitro*, the stem cells of embryonal carcinoma formed islets encircled by differentiated cells (Fig. 3a). In order to examine whether this segregation of the undifferentiated embryonal carcinoma cells and differentiated cells involves the process of selective adhesion of these cells, the following experiments were conducted.

AT805 cells dissociated into single cells by a routine procedure for cell passage using crude trypsin and EDTA were mixed with 129F or V79 cells similarly dissociated, and were placed in Falcon culture dishes (Falcon 3002) with DMEM containing 6% FCS. After 16 hr of culture, islets of embryonal carcinoma cells, well demarcated from fibroblastic cells, were formed (Fig. 3b). In another series of experiments, the mixed-cell suspensions were cultured on a gyratory shaker (at 80 rpm) in "bacterial" (nonadhesive) dishes (Falcon 1007) for 24 hr, and cell aggregates formed during this incubation were inoculated in Falcon culture dishes (3002). After the agregates became attached and spread on the dishes (within 18 hr), the cell constituent of each living aggregate was examined with an inverted phase-contrast microscope. In mixtures of AT805 and 129F, each aggregate consisted of a nearly pure population of either cell type (Fig. 3c). In mixtures of AT805 and V79, each aggregate usually contained both cell types, and embryonal carcinoma cells were always situated in the center of the aggregates (Fig. 3d). Since the segregation between embryonal carcinoma and fibroblastic cells occurred within a short culture period in these experiments, the clonal proliferation of cells is unlikely to be a main cause for the patched distribution in the heterologous cells. Perhaps, embryonal carcinoma cells and fibroblastic cells are able to recognize their own cell types in their adhesion. The role of CDS in this possible recognition was examined in the following experiments.

Cell-Type Specificity of Adhesion by Ca^{2+}-Dependent Sites

AT805 and F9 cells were dissociated with proC, and V79, W3, and 129F cells were dissociated with tryC so

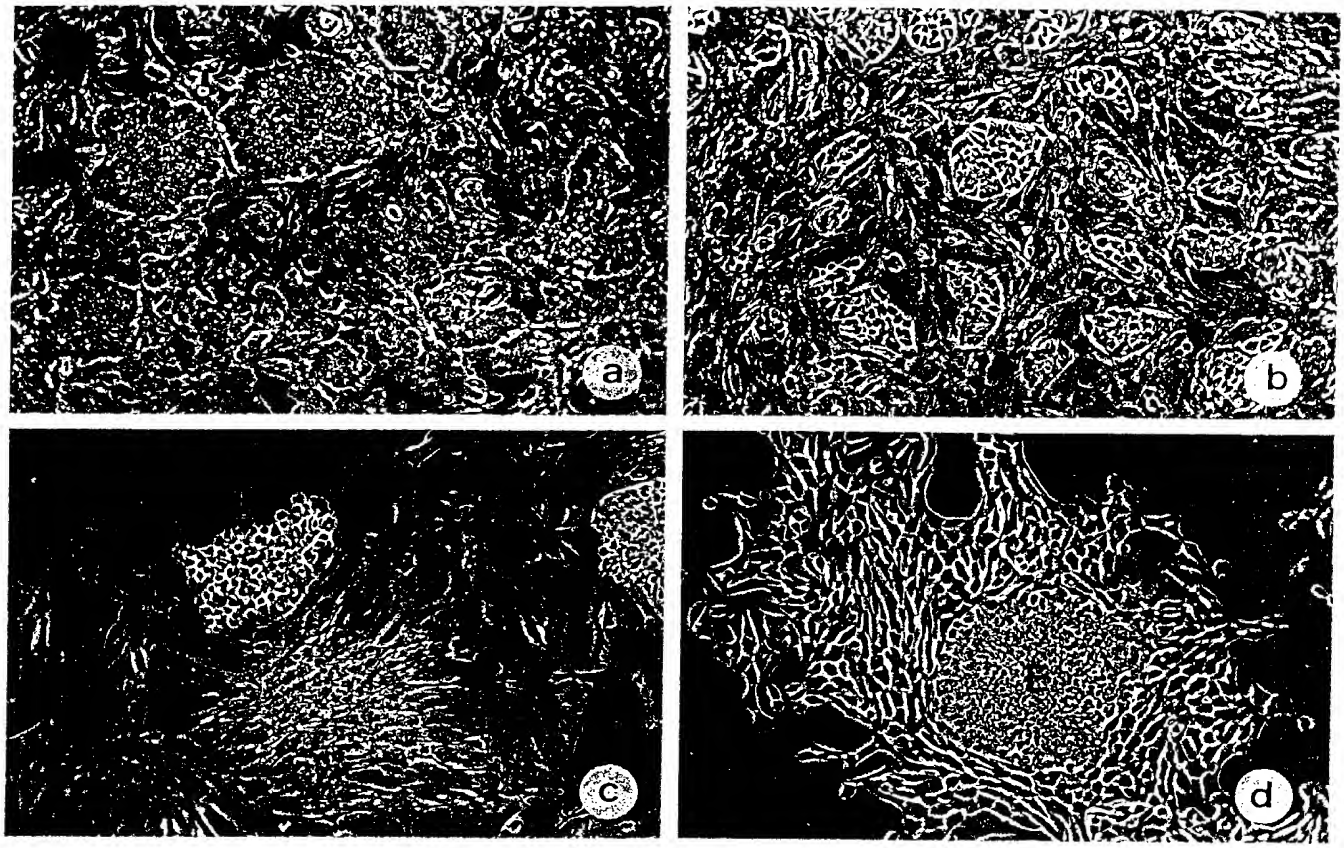

FIG. 3. Phase-contrast photomicrographs showing segregation of AT805 cells and differentiated cells. (a) Culture of AT805 tumor. Solid tumor formed after injecting AT805 cells into the peritoneal cavity of 129/Sv mouse was dissociated with trypsin and cultured for 7 days. (b) Mixture of AT805 and 129F cells cultured on the substrate for 16 hr. (c) Mixture of AT805 and 129F cells, and (d) of AT805 and V79 cells, cultured on the substrate for 18 hr after the aggregation for 24 hr. Each cell type was identified according to the morphological characteristics. Islets of undifferentiated cells of AT805 are indicated by E. ×100.

as to prepare cells having CDS but not Ca^{2+}-independent adhesion sites (CIDS). Among these five cell lines, cell mixtures of all possible binary combinations were made and incubated for 30 min on a gyratory shaker. Cells of either line in the mixed cell suspensions were previously stained with FITC.

In AT805 and F9 cell mixture, chimeric aggregates were formed in which cells of these lines were randomly mixed (Fig. 4). Similarly, in all combinations between V79, W3, and 129F cells, adhesion was nonselective (Fig. 4). However, when embryonal carcinoma lines were combined with fibroblast lines, cells of each line aggregated independently (Fig. 5). In some cases, embryonal carcinoma and fibroblastic cells were assembled into chimeric aggregates, yet they were always segregated within the aggregates, suggesting preferential adhesion between homotypic cells. Figure 6 summarizes the results obtained from all binary cell line combinations.

The rate of cell aggregation of each line somewhat varied with the experiments. The staining of cells with FITC sometimes caused a slight reduction in the aggregating capacity of the cells. Thus, there was the tendency that, in chimeric aggregates of stained and unstained cells, the number of stained cells was always smaller than that of the unstained cells. The basic pattern of selective or nonselective adhesion between different cell lines as described above, however, was not affected by minor changes or minor differences in the cell aggregation rate of any line.

One possible effect from using different proteases for dissociating cells on the segregation of different cell types in mixed aggregation was examined by mixing V79 cells dissociated with tryC and those with proC. No sign of segregation could be seen between these cells. In another experiment, V79 and AT805 cells, both dissociated with proC, were mixed together. Segregation occurred between these two cell types. Embryonal carcinoma cells dissociated with tryC were not used for this series of experiments, since completely single-cell suspensions were not obtained following this treatment.

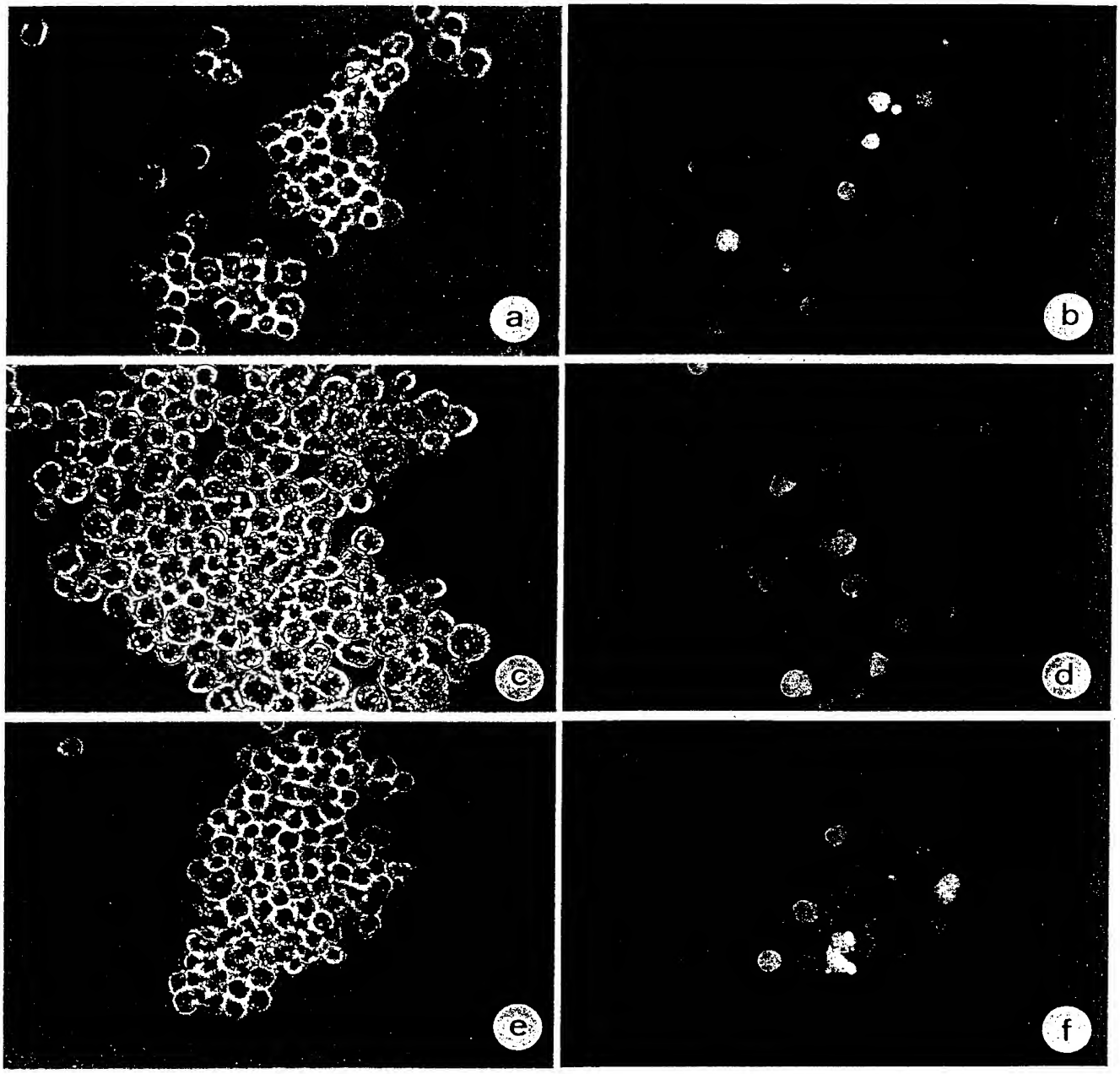

FIG. 4. Photomicrographs showing nonselective adhesion between homologous cell types. Mixed suspensions of two cell lines (usually 1 × 10^5 cells each line) dissociated with proC or tryC under the same conditions as in Table 1 were incubated for 30 min to bring about cell aggregation. In suspensions of 129F cells, 2 × 10^4 cells of this line were combined with 1 × 10^5 cells of other lines, since the size of 129F cells is a few times larger than the others. (a and b) AT805* and F9, (c and d) V79* and 129F, and (e and f) W3* and V79 (cell lines marked with an asterisk were stained with FITC). (a, c, and e) Phase-contrast micrographs; (b, d, and f) fluorescence micrographs of the same field as in a, c, and e, respectively. × 200.

Molecular Basis of Cell-Type Specificity of Ca^{2+}-Dependent Sites

Fab fragments of antibodies against tryC-dissociated F9 cells (anti-tCF9 Fab) completely inhibited the aggregation of F9 and AT805 cells dissociated with tryC or proC (Table 2), depending on the concentration of the Fab (Fig. 7). This inhibitory effect was abolished by absorbing the Fab with F9 cells dissociated with tryC or proC but not with those dissociated with tryE

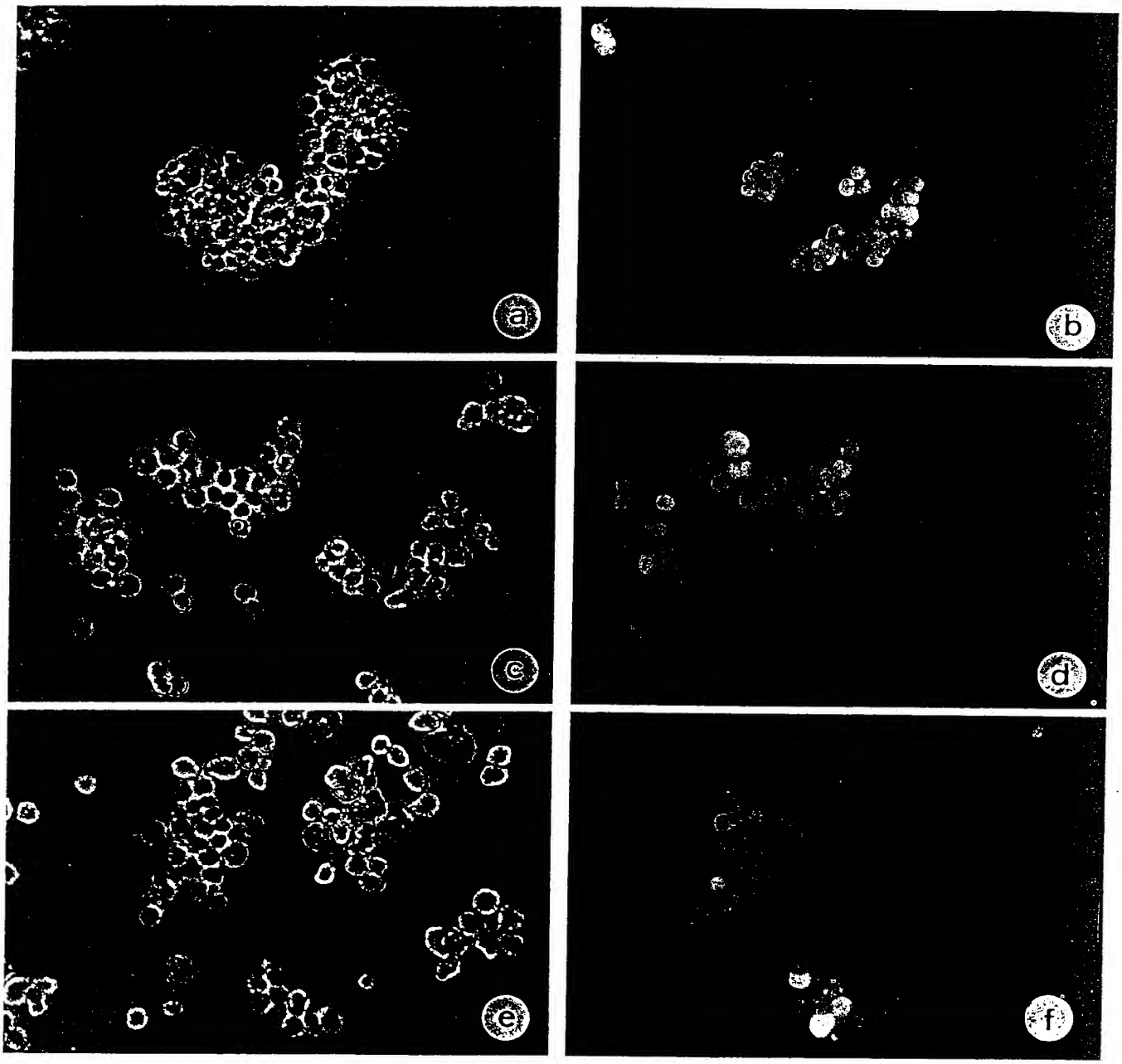

FIG. 5. Photomicrographs showing selective adhesion between heterologous cell types. The experimental conditions are the same as in Fig. 4. (a and b) AT805 and V79* (c and d) F9* and V79, and (e and f) F9* and 129F. (a, c, and e) Phase-contrast micrographs; (b,d, and f) fluorescence micrographs of the same field as in a, c, and e, respectively. × 200.

or proE (Fig. 8). These results clearly indicate that the inhibition of aggregation by anti-tCF9 Fab is due to the binding of the Fab with molecules present specifically in the cells with CDS.

The Ca^{2+}-dependent aggregation of V79, W3, and 129F cells dissociated with tryC was not or little inhibited by anti-tCF9 Fab in concentrations which completely inhibited the aggregation of F9 cells (Table 2). These fibroblasts did not absorb the aggregation-inhibitory activity of anti-tCF9 Fab (Table 2).

In order to obtain information on the molecular nature of the CDS of embryonal carcinoma and fibroblastic cells, the surface proteins of these cells dissociated with proteases in the presence and absence of

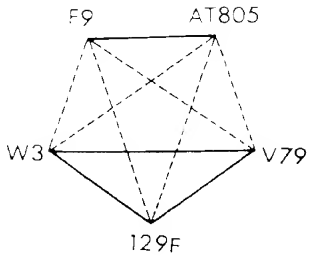

FIG. 6. Summary of results on the selective and nonselective adhesion between various cell lines. Cell lines connected by solid lines did not segregate, while cell lines connected by dashed lines segregated.

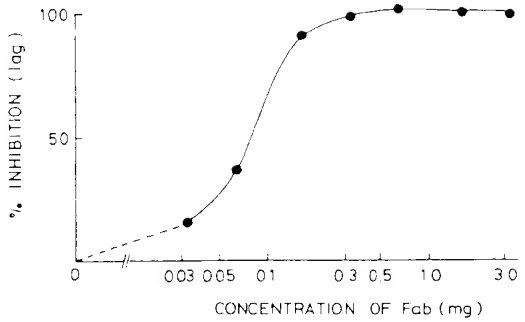

FIG. 7. Inhibition of the Ca^{2+}-dependent aggregation of F9 cells with anti-tCF9 Fab. Cells (1×10^5) dissociated with 0.01% Pronase containing 1 m*M* Ca^{2+} (proC) were suspended in 0.5 ml of HMF containing various amounts of Fab. These suspensions were preincubated at 4°C for 10 min to allow the binding of Fab with antigens, and then incubated at 37°C for 60 min on a gyratory shaker. The degree of cell aggregation inhibition is represented by the index *Iag*, defined as $Iag = [(N_t(c) - N_t(0))/(N_0 - N_t(0))] \times 100$ (%), where $N_t(c)$ is the total number of particles at incubation time t in the presence of Fab at concentration c (for details see Urushihara *et al.* (1979)).

Ca^{2+} were radioiodinated and their patterns in SDS-polyacrylamide gel (7.5 or 15% polyacrylamide) electrophoresis were compared (Fig. 9). Out of a number of radioiodinated components in V79 cells, a component with a molecular weight (MW) of 150,000 (p150K) was detected only in cells with CDS, as described previously (Takeichi, 1977). No other radioiodinated components could be correlated with CDS. Similarly, in W3 and 129F, p150K was found after dissociation with tryC but not with tryE. The same was observed in L cells, although the intensity of the p150K band in the autoradiograms of gels was much weaker here than in other cell lines, consistent with the low capacity for Ca^{2+}-dependent aggregation of these cells.

There seemed to be other differences in the patterns of radioiodinated bands in the autoradiograms between tryC- and tryE-dissociated cells of these murine fibroblast lines, particularly 129F. These differences, however, were not commonly found in all cell lines. p150K was resistant to vaious proteases, Pronase, papain, and proteinase K, as well as trypsin, only when Ca^{2+} was present. Therefore, there is a clear correlation between the presence of p150K and the presence of CDS on the cell surfaces.

In the case of embryonal carcinoma cells, a component with MW of 140,000 (p140K), instead of p150K, was specific to AT805 and F9 cells dissociated with tryC and proC, and was not detectable in those cells dissociated with tryE and proE (Fig. 9). No radioiodinated component other than p140K could be correlated with CDS in embryonal carcinoma cells.

TABLE 2
INHIBITION OF CELL AGGREGATION OF VARIOUS LINES WITH ANTI-tCF9 Fab

Fab (0.33 mg/ml)	Percentage inhibition (*Iag*)[a]				
	F9	AT805	V79	W3	129F
Fab unabsorbed	102	103	−8.7	11.8	−1.6
Fab absorbed with[b]					
F9 cells	1.5				
V79 cells	99				
W3 cells	105				

Note. F9 and AT805 cells were dissociated with proC, and V79 and W3 were dissociated with tryC under the same conditions as in Table 1.

[a] Aggregation was measured following the incubation of cells with and without Fab for 60 min, and *Iag* was calculated, as described in Fig. 7 legend. Data represent the average of duplicate determinations.

[b] Fab (0.33 mg) in 1 ml HMF was absorbed with 4×10^7 cells of each line as described in Fig. 8 legend.

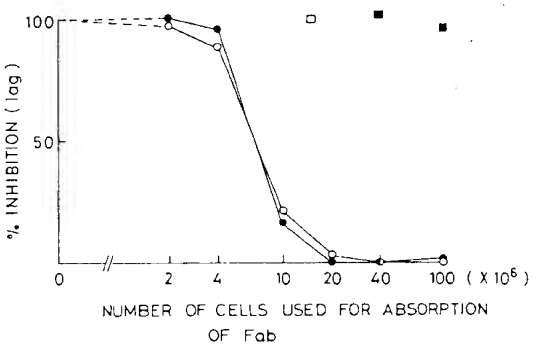

FIG. 8. Absorption of the aggregation-inhibitory activity of anti-tCF9 with F9 cells dissociated by different treatments. To bring about absorption, 0.33 mg Fab in 1 ml HMF was incubated with various numbers of F9 cells dissociated with 0.01% trypsin containing 1 m*M* Ca^{2+} (tryC) (●), 0.01% trypsin containing 1 m*M* EGTA (tryE) (■), 0.01% Pronase containing 1 m*M* Ca^{2+} (proC) (○), or 0.01% Pronase containing 1 m*M* EGTA (proE) (□), in an ice bath for 60 min with occasional shaking. To these absorbed Fab preparations, F9 cells (1×10^5/0.5 ml) dissociated with proC were added and incubated for 60 min for aggregation. *Iag* was calculated as described in Fig. 7 legend.

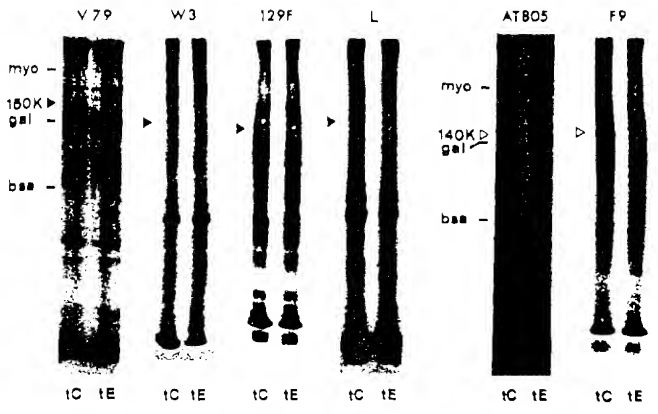

FIG. 9. Autoradiograms of SDS-polyacrylamide gels following electrophoresis of lysates of ^{125}I-labeled cells dissociated with tryC (tC) and tryE (tE) under the same conditions as in Table 1. Gels shown here contained 7.5% acrylamide. ▶, p150K; ▷, p140K. Molecular weight markers: myo, mouse skeletal myosin; gal, β-galactosidase from *Escherichia coli*.; bsa, bovine serum albumin. Positions of these markers were shown only for V79 and AT805.

DISCUSSION

The present study shows that embryonal carcinoma cells adhere to each other through Ca^{2+}-dependent sites (CDS) similar in nature to corresponding sites in fibroblastic cells (Takeichi, 1977; Urushihara *et al.*, 1979). The relative difficulty in dissociating embryonal carcinoma cells with trypsin may be due to the presence of trypsin-resistant intercellular junctions, such as tight junction (Dunia *et al.*, 1979) in embryonal carcinoma cells, which are perhaps absent in fibroblastic cells. The use of Pronase for the dissociation of embryonal carcinoma cells was effective to overcome this problem. Ca^{2+} protected the CDS from inactivation by treatment with a variety of proteases.

In the examples so far reported, the cell-to-cell adhesion by CDS was neither cell-type specific nor species specific. Chick neural retina cells (Takeichi *et al.*, 1979) or human retinoblastoma cells (Uéda *et al.*, 1980) adhered to Chinese hamster V79 fibroblasts by CDS without any selectivity. The present observations of segregation between embryonal carcinoma and fibroblastic cells in the aggregation experiments constitute the first example indicating the involvement of CDS in specific cell adhesion.

The selective distribution of embryonal carcinoma and differentiated fibroblastic cells in monolayer cultures may probably be ascribed to the cell-type specificity of adhesion by CDS. In cultures containing a mixed population of these two cell types, each cell recognizes homotypic cells by CDS, and establishes a more stable adhesion to the homotypic cell than to the heterotypic cell, although another nonselective adhesion mechanism may cooperate in the binding of heterologous cell types. Ca^{2+}-independent adhesion sites (CIDS) can contribute to either selective or nonselective adhesion between embryonal carcinoma and fibroblastic cells, as found in other systems of heterotypic cell adhesion (Takeichi *et al.*, 1979; Uéda *et al.*, 1980).

Embryonal carcinoma cells metabolically cooperate between themselves but not with differentiated cells (Nicolas *et al.*, 1978; Lo and Gilula, 1980). This observation is quite consistent with our previous and present results. Atsumi and Takeichi (1980) reported that the adhesion between cells mediated by CDS is prerequisite for the formation of gap junctions which are known to be necessary for establishing metabolic cooperation. Gap junctions, therefore, may not be formed between embryonal carcinoma and some differentiated cells such as fibroblasts, which can not be linked together by CDS as shown in the present paper.

The effect of anti-tCF9 Fab to inhibit Ca^{2+}-dependent aggregation of embryonal carcinoma cells was abolished only by absorbing with embryonal carcinoma cells which had been dissociated leaving CDS intact. This strongly suggests that the Fab inhibits the cell aggregation by the direct binding with cell adhesion molecules, but not by binding with other cell surface antigens, although we can not totally exclude the latter possibility.

The selective inhibition of Ca^{2+}-dependent aggregation of embryonal carcinoma cells with anti-tCF9 Fab probably indicates that molecules involved in CDS in embryonal carcinoma cells are distinct from those in fibroblastic cells. Indeed, a search for molecules in CDS by electrophoresis of radioiodinated cell surface proteins revealed that components with different molecular weights, p140K and p150K, correlated with the CDS of embryonal carcinoma and fibroblastic cells, respectively. No direct evidence of the functions of these candidate components in cell adhesion has been obtained yet. To achieve this goal, we are attempting to prepare specific antibodies against each component. Another approach will be to purify the antibodies specific to CDS from anti-tCF9 by absorbing other nonspecific antibodies with the cells from which CDS has been removed, such as proE-dissociated F9 cells. By using these antibodies, the molecular properties of CDS would be finally clarified.

Effects of rabbit antibodies raised F9 cells (anti-F9) and other teratocarcinoma cells on the morphology of mouse cleaving embryos and embryonal carcinoma cells have been extensively studied (Kemler *et al.*, 1977; Johnson *et al.*, 1979; Ducibella, 1980; Hyafil *et al.*, 1980; Nicolas *et al.*, 1981). These antibodies and their Fab fragments prevent the compaction of blastomeres at the eight-cell stages of mouse embryo and also induce the

rounding up (or decompaction) of embryonal carcinoma cells in monolayer culture. The decompacting activity of anti-F9 was absorbed only with teratocarcinoma cells and their early differentiated derivatives, but not with several kinds of differentiated cells such as fibroblasts and myoblasts. The targets of the anti-F9 inducing the decompaction of cells thus show a cell-type specificity similar to that of CDS in embryonal carcinoma cells. The compaction of blastomeres and embryonal carcinoma cells in culture depends on the presence of Ca^{2+} (Ducibella and Anderson, 1975, 1979; Nicolas *et al.*, 1981), suggesting the possibility that the compaction requires the cell adhesion by CDS. CDS, therefore, could be one of the targets of anti-F9. The glycoprotein, which was shown to absorb the above effect of anti-F9, identified by Hyafil *et al* (1980) might have some relevance to CDS in embryonal carcinoma cells.

The relationship of cell adhesion molecules indicated in the present study with molecules implicated in cell adhesion according to the reports of other people using neural retina cells (Hausman and Moscona, 1976; Thiery *et al.*, 1977), liver cells (Bertolotti *et al.*, 1980), ascites hepatoma cells (Kudo *et al.*, 1976), and teratocarcinoma (Oppenheimer and Humphreys, 1971; Grabel *et al.*, 1979) remains to be solved.

There are many similarities between embryonal carcinoma cells and the embryonic cells at early developmental stages in regard to cell surface properties including intercellular adhesion mechanisms, as suggested by the effect of anti-F9 (Jacob, 1979). It can be speculated that the cells at early developmental stages of mouse embryo possess embryonal carcinoma type CDS (p140K) for their mutual adhesion, and lose it with their differentiation, acquiring the new type of cell adhesion molecules such as fibroblast type CDS (p150K). This kind of conversion of cell adhesion molecules with cell differentiation may play important roles for segregation between different cell types in mammalian development. The present findings thus open a new approach elucidating the molecular mechanisms of animal morphogenesis.

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